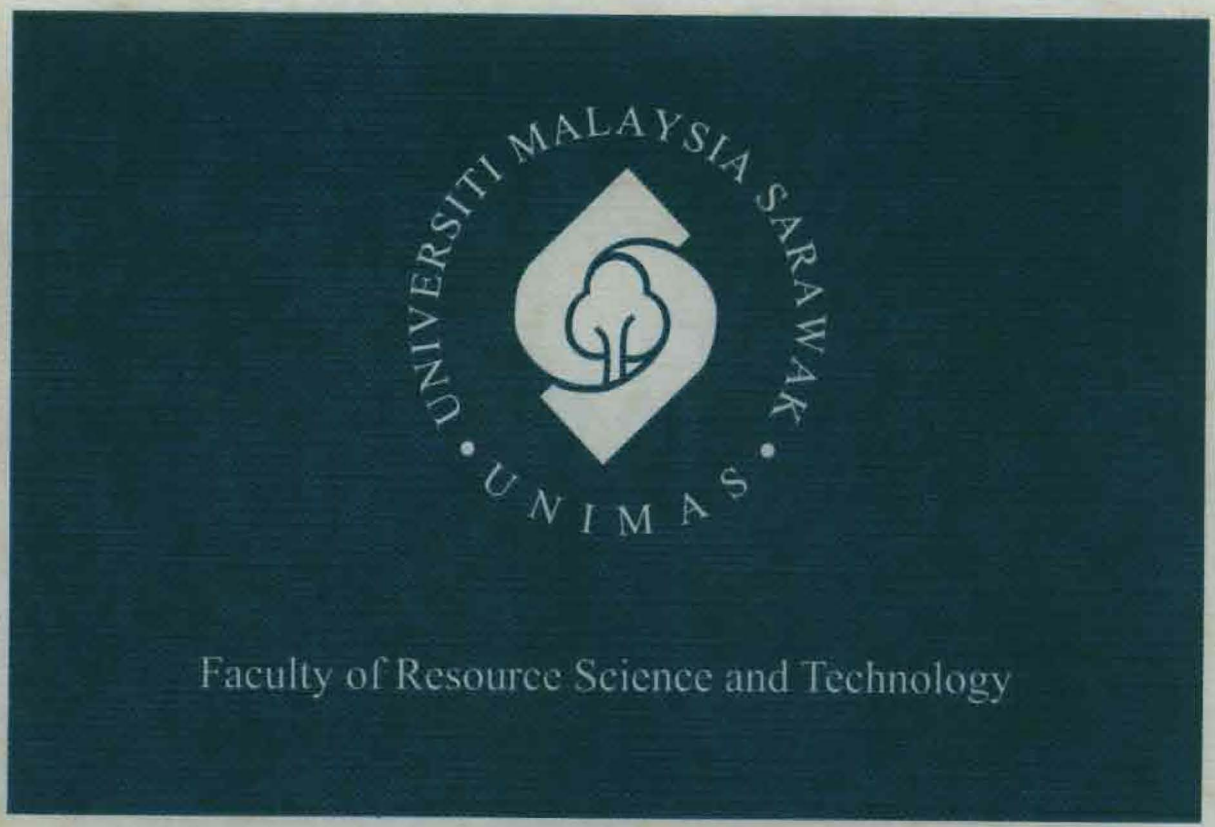

CHEMOTAXONOMIC STUDY OF CUTICULAR HYDROCARBON FROM CURCULIONID BEETLES (O: COLEOPTERA, F: CURCULIONIDAE)

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Bachelor Science with Honours
(Department of Zoology)
2008

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This project report is submitted in partial fulfilment of the requirements for the Degree of

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DECLARATION

No portion of this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

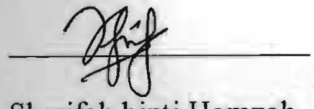

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

A chemotaxonomic study of cuticular hydrocarbon from Curculionid beetles

(O:Coleoptera, F:Curculionidae)

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ABSTRACT

Taxonomy based on differences in morphological characters and biosystematics studies have been widely used to classify beetles. However, in this research, different approach was used by investigating the variation of hydrocarbon pattern in curculionid beetles from family Curculionidae by using cuticular hydrocarbon. The cuticular lipids were extracted and evaluated from four different species of curculionid beetles which were *Rhynchophorus schach, Odoiporus longicollis, Sitophilus zeamays* and *Sitophilus oryzae.* The cuticular hydrocarbon of curculionid beetles were extracted using n-hexane and analyzed through Gas Chromatoraphy-FID. Sixteen types of n-akanes were found on entire species and the hydrocarbon chain length were varied from C_{20} to C_{35}. Odd carbon chain of n-alkanes were dominated in proportion of n-alkane in the entire species. N-tricosane, n- hezacosane, n- heptacosane, n- octacosane, n-hentriacontane were majority carbon present in all the selected curculionid beetles.

Key words: Cuticular hydrocarbon, Curculionidae, n-hexane, Gas Chromatography-FID

ABSTRAK

Taksonomi berdasarkan perbezaan melalui ciri morfologi dan biosistematik telah digunakan secara meluas untuk mengklasifikasikan kumbang. Namun begitu, kajian ini menggunakan pendekatan yang berlainan dengan menyelidik corak hidrokarbon pada kumbang 'curculionid' dari Famili 'Curculionidae' menggunakan kutikel hidrokarbon. Kutikel lipids diekstrak and dikira daripada empat spesis yang berbeza iaitu *Rhynchophorus schach, Odoiporus longicollis, Sitophilus zeamays* dan *Sitophilus oryzae.* Kutikel hidrokarbon dari kumbang 'curculionid' diekstrak menggunakan n-heksan dan dianalisis menggunakan Gas Kromatografi- FID. Enam belas jenis n-alkanes telah dikenal pasti pada keseluruhan spesis dan rantai hidrokarbon untuk kumbang 'curculionid' berada diantara dari C_{20} sehingga C_{35}. Pecahan n-alkana dalam kutikel hidrokarbon kumbang 'curculionid' adalah dimonopoli oleh rantai karbon nombor ganjil. N-trikosan, n- hezakosan, n- heptakosan, n- oktakosan, n-hentriakontan adalah majoriti karbon yg hadir pada kesemua spesis pada kumbang 'Curculionid' yang dipilih.

Kata kunci : Kutikel hidrokarbon, Curculionidae, n-heksana, Gas Kromatografi- FID.

1.0 INTRODUCTION

Family Curculionidae which is also known as true weevil belongs to the order Coleoptera is a vast group where it is estimated to exceed 60,000 species worldwide (Hill and Abang, 2005). The adult curculionid beetles can be recognized by having distinctive geniculate, cubbed antennae, a long rostrum which bear the mouthpart distally, with their body is covered partially or completely with scales and bristle.

Curculionid beetle is chosen for this study due to the universality of the family and also the ease of sample collection. This is because of its abundance in the tropics as compared to temperate regions (Morris, 1991). Besides, it can be found in stored products as mostly worlds weevils are pests (Baker and Nelson, 1981).

On a regular basis, morphological characteristic and dichotomous keys had been used to identify species for insect systematic (Page *et al.* 1997). However, the problems and the difficulty encountered through the identification of morphological characters and also the cost through chromosomal analysis technique, had led to other methods such as chemotaxonomic approach using cuticular hydrocarbon.

The cuticular hydrocarbon analysis will be performed to investigate whether cuticular hydrocarbon can be used for species recognition of curculionid beetles and also to initiate their cuticular hydrocarbon profile. The simplification, inexpensiveness and the time-efficient properties of the analysis made it a recommended alternative option for species recognition of beetles (Bosorang, 2006). Thus, this approach can offer an alternative mean to identify beetles besides using morphological keys and molecular genetic.

Cuticular hydrocarbon study on species-specificity had been conducted on termites (Howard *et al* ,1982), beetles (Page *et al.*, 1990), flies (Urech *et al.*, 2005) and also grasshoppers (Chapman *et al.*, 1995). Examination of cuticular lipids of some species had earlier revealed significant differences in composition of hydrocarbon (Page *et al.*, 1992). To date, there were only few studies of the cuticular hydrocarbon of beetles and weevils (Baker and Nelson, 1981; Bosorang, 2007) especially in curculionid weevils. Yet, the cuticular hydrocarbon study was not widely applied in Malaysia and mostly applied in foreign countries.

Furthermore, the information on qualitative and quantitative data of cuticular variation are still lacking among beetles particularly in Sarawak. Therefore, this study will attempt to create cuticular outline for selected curculionid beetles of Sarawak particularly from Kota Samarahan.

Through this study, it is hoped that it can provide answers to a few questions such as:

1. Are there any significant differences in the composition of cuticular hydrocarbon mixtures between species?
2. Are there any significant differences in the composition of cuticular hydrocarbon mixtures between different genders?

Thus, the main objectives of this study are:

- To extract, characterize and identify hydrocarbon fractions of cuticular lipid of curculionid beetles (Coleoptera) by using gas chromatography.
- To compare cuticular hydrocarbon composition between different genders of selected species of curculionid beetles.
- To compare the cuticular hydrocarbon composition between four different species of curculionid beetles.
- To explore the usefulness of cuticular hydrocarbon profiles as chemotaxonomic characters for curculionid beetles.

2.0 LITERATURE REVIEW

2.1 Chemotaxonomy on Cuticular Hydrocarbon

Chemotaxonomy, also called chemosystematics, is a classification of organisms according to differences in their biochemical makeup (Shelby, undated). Frequently, when the morphological characteristic cannot be relied upon to identify a species, the taxonomist have to compare the components present on the species (Shelby, undated; Page *et al.*,1990)

Whitlow (2003) reported there were four classes of cuticular hydrocarbon which includes n-alkanes, olefins, monoethylalkanes and polyethylalkanes. Singer (1988) stated the advantage of cuticular hydrocarbon as a measured standard for systematic classification of insects due to the presence of wax coatings, difference of composition on other species, the simplicity of samplings and the presence of analytical tools for statistical data analysis.

The cuticle of insect is coated with a mixture of organic compounds which are alcohols, fatty acids, esters, ketones, glycerides, sterols, aldehydes and hydrocarbons (Blomquist *et al.* 1987). However, Blomquist *et al.*(1987) states that mostly hydrocarbon compounds in the cuticular lipids with 90 percent predominated. Besides, the cuticle or wax layer served as a shield for from abrasion, controlling dehydration, chemical and other microorganism (Hadley, 1985).

A taxonomic study on cuticular hydrocarbon is one of available ways to identify organisms which were specifically classified according to lipid sequences. Cuticular hydrocarbon analysis has been applied to many insect and there were previous studies done on insect

such as beetles (Page *et al.*, 1997), termites (Etges and Ahrens, 2001; Dronnet *et al.*, 2006), ants (Lomellen *et al.*, 2006), fly (Urech *et al.*, 2005) and also hymenoptera (Howard, 2001).

According to Urech *et al.* (2005), when the two species is difficult to separate by morphological characters, cuticular hydrocarbon analysis can be one of the alternative methods to differentiate between the two species. Besides, it can also be applied to species where their dichotomous keys are so few and poorly developed for the accurate identification of species (Page *et al.*, 1990).

Etges and Jackson (2001), stated the quantitative difference in amount of hydrocarbon is due to the chemical signatures that have evolved within and between species. According to Etges and Ahrens (2001) based on their studies on termite species, cuticular hydrocarbon serves as part of mate recognition system.

There was a study done on cuticular hydrocarbon of adults' cowpea weevil, *Callosobruchus macultus* to investigate the variation between species genders. Baker and Nelson (1981) revealed that there were no differences found in hydrocarbon profile between males and females of adult cowpea weevils. They also pointed out that hydrocarbon compound is the major compound in cuticular lipids and it is constituted of four homologous series of alkanes. The four homologous series of alkanes which were identified by gas chromatography - mass spectrometry (GC-MS) analysis were n-alkanes, internally branched monomethylalkanes, terminally branched monomethylalkanes, and internally branched dimethylalkanes. The study resulted that part of hydrocarbon is mostly

made up by mono and dimethyl branched-chain alkanes and there were no presence of alkenes during analysis of the cowpea weevils.

Furthermore , study of cuticular hydrocarbon of bark beetles (F: Scolytidae) done by Page *et al.* (1990) showed that there were five classes of hydrocarbon present which are n-alkanes, internally branched monomethylalkanes, terminally branched monomethylalkanes, internally branched dimethylalkanes followed by alkenes. The study state that 3,7 dimethylakanes was the most suitable hydrocarbons to use to identify between two different species of bark beetles, which are *Dendroctonus ponderosae* and *D. jeffryi.*

Besides, Page *et al.* (1990) claimed that the cuticular hydrocarbon analysis does not affect the condition of the sample. They also suggested that the distinct feature of cuticular hydrocarbon mixture can be useful for determination of systematic relationship.

While Howard (2001), on his previous study of cuticular hydrocarbons of adult *Pteromalus cerealellae* (Hymenoptera: Pteromalidae) and two larval hosts, Angoumois Grain Moth (Lepidoptera: Gelechiidae) and Cowpea weevil (Coleptera: Bruchidae) stated differences on the cuticular hydrocarbon mixtures between two different host species. This is because, parasitoids of stored-product pests use cuticular hydrocarbons as major species and in gender-recognition cues, and moth and beetle hosts differ greatly in their hydrocarbon profiles (Howard , 2001).

Moreover, according to Chapman *et al.* (1995), there were slight differences between immature and adult grasshopper. However, it does not influence the construction of hydrocarbon profile. In his previous study, Chapman *et al.* (1995) revealed that different

gender does not affect the abundance of the hydrocarbon. It also stated that, there were variation between species on grasshopper and it can be determined by using hydrocarbon analysis. Therefore, Chapman *et al.* (1995) conclude that taxonomic study of certain species of grasshopper can potentially use the cuticular hydrocarbon analysis however it must be from various species and various habitats.

Jones *et al.* (1997) stated that cuticular lipid can be a taxonomic marker where there is a suggestion on the similarity of cuticular hydrocarbons compositions which it reflects in the taxonomic grouping of locusts.

A study on bark beetles, Scolytidae conducted by Page *et al.* (1990) stated that there were no qualitative differences between genders in hydrocarbon composition. The odd-number alkanes were dominant compound (*n*- tricosane, *n*- pentacosane, *n*- heptacosane and *n*-nonacosane) constitutes 60% of total of hydrocarbons components. Besides, the differences of species in geographic location and host tree did not slightly effect the hydrocarbon composition in *D. penderosae, D. jeffreyi, D. brevicomis* and *D. frontalis* .

However, Baker and Nelson (1981) stated that the weevils, *C. maculutus* has high amount of total lipid if reared at high temperatures. It is shown that the geographic location has potential influence in lipid composition in certain species.

Cuticular lipids of adult grasshopper, *Schistocerca hoshone* from six different localities in southeastern (Portal and Tacna) and southwestern (Arizona) of United State of America were analyzed by Chapman *et al.* (1995). The studies showed the relation of the cuticular lipid variation to population differences and environmental variables. The study suggested

that grasshopper from high summer temperature area have high proportion of n- alkanes. In addition, food types of insect reared in laboratory have minor effect in hydrocarbon compound compared from different population.

Chapman *et al.* (1999) analyzed cuticular lipid of three species of adult grasshoppers from the Galapagos islands, Ecuador. Fifty percent of hydrocarbon comprises of n-alkanes ranged between C_{23} until C_{37} where heptacosane, C_{27} is the most abundant compound. The difference of n-alkanes composition can proclaim if the two species are connected. But, it cannot be applied to methylbranched alkanes as it was considerably different between two species.

While research on cuticular hydrocarbon of eight species of cone beetles by Page *et al.* (1990) revealed that the composition of the n-alkanes in all species is a continuous series from n-heneicosane (C_{21}) to C_{31} with the dominating compounds were C_{23}, C_{25} and C_{27}. Besides, all species has measurable quantities of n-alkanes of even numbered chain length from C_{22} to C_{28}, which C_{24} and C_{26} are the most abundance compounds.

3.0 MATERIALS AND METHODS

3.1 Sample Collection.

The adult curculionid beetles were collected using light trap, beating trays and pit-fall trap. The traps were set up around Unimas campus and around Samarahan, Sarawak. Other than that, some curculionid beetles like *Odoiporus longicollis* were collected by hand at banana tree.

3.2 Species Identification.

The collected curculionid beetles were identified by referring to available identification books such as Tung (1983) and also to the voucher specimens at Museum of Zoology, UNIMAS.

3.3 Extraction and Fraction of Cuticular Hydrocarbons.

Curculionid specimens were defrozened to ambient temperature prior to extraction of cuticular lipids. The cuticular lipid was extracted by immersing the insect specimen in 10 mL of hexane for approximately 10 minutes duration (Page *et al.,* 1990). Fifty microleads of 50 ppm mixture of octadecene and eicosene was spiked into the extracts. Octadecene and eicosene acted as internal standard. The extracts were then introduced on the top of column chromatography packed with activated Biosil A (silica gel, 100-200 mesh) in order to remove any of interferences that presence in the extracts. The column was eluted with 20 mL hexane and the eluant was collected in 25 mL capacity pear shape

flask. The sample was then evaporated to approximately 2-3 mL using vacuum rotary evaporator. The extract was then transferred to 5 mL vial and evaporated to dryness by blowing with a gentle stream of nitrogen gas. Prior to gas chromatographic analysis, the extract was redissolved with 200 µL of n-hexane.

3.4 Voucher Specimen.

The extracted insect specimens were deposited in Zoology Museum of Faculty Resource Science and Technology, UNIMAS. These specimens were stored in 70% ethanol for later species identification based on morphological characteristics and also served as voucher specimens.

3.5 Gas Chromatographic Analysis.

Gas chromatographic analysis of the extracts was performed on a Hewlett Packard 5790A gas chromatograph with flame ionization detector (GC/FID) equipped with a 25 m x 0.2 mm x 0.33µm (film thickness) DB-5 capillary column. The oven temperature was initially set at 50°C for 5minutes and the ramped to 310°C at the rate of 6.5°C/min. The final temperature was hold for 20 minutes. The temperature for injector and detector were 250°C and 280°C, respectively. Individual n-alkane was identified by comparing the retention time acquired with the retention time of particular n-alkane in mixture of n-alkane standard (C_{10}-C_{36}).

3.6 Qualitative Analysis.

The peak areas for external standard of n-alkanes were used in identification of n-alkanes extracted. From the GC data, an analysis of the peak areas was manually done by comparing the external standard with base line for each peak areas. For each species, the peak areas were determined based on the retention times (Appendix 8).

For external standard of n-alkanes, the mixed hydrocarbons 50 ppm were used where the mixture includes n-nonane (C_9), n- decane (C_{10}) , n- dodecane (C_{12}) , n- tetradecane (C_{14}), hexadecane(C_{16}), n-octadecane (C_{18}), n-eicosane (C_{20}), n- decosane (C_{22}), n-tetracosane($_{24}$), n- hexacosane(C_{26}), octacosane(C_{28}), n- triacontane (C_{30}), n-dotriacontane(C_{32}) , n- tetratriacontane (C_{34}) and n- hexatriacontane (C_{36}) (Figure 1).

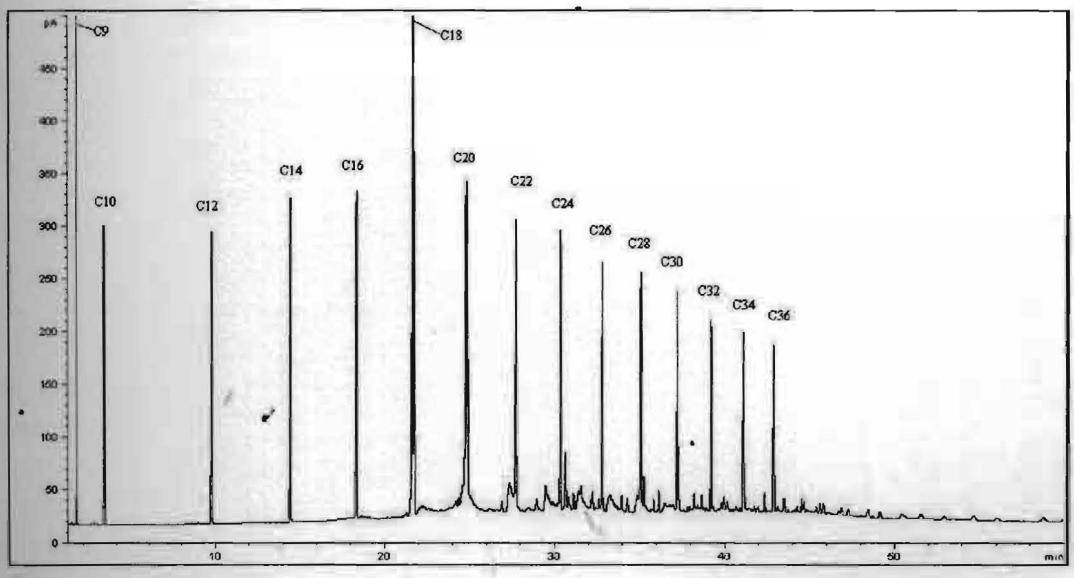

Figure 1: Peak area for external standard n-alkanes used in identification of n-alkane extracted.

3.7 Quantitative Analysis.

The peak areas from gas chromatogram of standard n-alkanes were used for quantitative analysis of n-alkanes in the extracts. The areas of n-alkane peaks were used as parameter for quantitative analysis. The amount of individual n-alkane was calculated based internal standardization method using the following equations:

$$\text{Amount of analyte X (ng)} = \frac{\text{RRF} \times (\text{Area of analyte X}) \times (\text{Amount of IS})}{\text{Area of IS}}$$

Where,

$$\text{Relative Response Factor (RRF)} = \frac{[\text{X}]}{\text{Area of X}} \times \frac{\text{Area of IS}}{[\text{IS}]}$$

Then, percentage amount of each analyte was calculated using the following equation:

$$\% \text{ concentration amount of analyte} = \frac{\text{Amount of analyte X}}{\text{Total Amount of Analyte}} \times 100$$

Whereas, CPI (Carbon Preference Index) was used to identify either odd or even number of hydrocarbon chain length were dominated in the sample. When the calculated value is more than 1.0, it indicates the odd number or when less than 1, the even number hydrocarbon chain was dominated.

Carbon Preference Index, CPI =

$$\frac{1}{2}\left(\frac{C25 + C27 + C29 + C31 + C33}{C26 + C28 + 30 + C32 + C34}\right) + \left(\frac{C25 + C27 + C29 + C31 + C33}{C24 + C26 + C28 + 30 + C32}\right)$$

3.7 Statistical Analysis

SPSS (Statistical Package for Social Sciences) were used to analyze the data obtained by using Principal Component Analysis (PCA) on fully Factorial with analysis of variance (ANOVA) option defining all the identified lipid components.

Cluster analysis (Krebbs, 1999) was performed using Multi Variate Statistical Package (MVSP) where Jaccard Index was used as distance measure. It is focus on qualitative differences in cuticular hydrocarbon pattern and if a specific chemical present or not. The cluster analysis was group according to the similarities in cuticular hydrocarbon compound and it is hoped to show the relationship between species.

4.0 RESULTS

4.1 Distribution pattern of n-alkanes in selected curculionid beetles.

N-alkanes data were obtained from the pooled samples consist of both female and male individuals. Qualitative and quantitative analyses were performed on n-alkanes in extracts from cuticular lipids of curculionid beetles.

The n-alkanes identified in the extract for cuticular lipid of four selected species of curculionidae are shown in Table 1. From Table 1, the fraction and the pattern of the n-alkanes compound present are varied from species to species. The n-alkanes identified consist of homologous series range between C_{20} to C_{35}.

Only sixteen hydrocarbons component were identified by GC-Fid from the four selected species; *Rhynchophorus schach, Odoiporus longicollis, Sitophilus zeamays* and *Sitophilus oryzae*. N-alkanes with carbon number less than 20 were not detected in the extracts of Curculionid beetles. The n-alkanes identified in cuticular wax for curculionid beetles are n-alkanes with carbon number more than 20 and these n-alkanes can be categorized as high molecular hydrocarbons.

Analysis of variance signified that there was a significance difference between proportion of n-alkanes among species (p value = 0.005, less than 0.05 [ANOVA Table, Appendix 7.a]). The fewer p value < 0.05 implied that there was variation of n-alkanes pattern and composition between species.